Relationships between the structural characteristic of curcumins that affect cell proliferation of hepatocarcinoma cells.

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Relaciones entre las características estructurales de las curcuminas que afectan la proliferación celular de las células de hepatocarcinoma.

Relacions entre les característiques estructurals de les curcumines que afecten la proliferació cel·lular de les cèl·lules de l'hepatocarcinoma.

RECEIVED: 14 SEPTEMBER 2022; ACCEPTED: 6 JUNE 2023; DOI: https://doi.org/10.55815/417980

ABSTRACT

Relationships between the structural characteristic of curcumin and dimethoxycurcumin and their antitumoral activity were studied. Treatment of HepG2 cells for 24 h with the curcumin and dimethoxycurcumin resulted in apoptosis induction and dose-dependent inhibition of cell proliferation. The calculated docking and the DFT method, suggest a structure-activity relationship between the activities of dimethoxycurcumin and curcumin structure and the apoptosis in HepG2 cells.

RESUMEN

Se estudiaron las relaciones entre las características estructurales de la curcumina y la dimetoxicurcumina y su actividad antitumoral. El tratamiento de las células HepG2 durante 24 h con curcumina y dimetoxicurcumina dio como resultado la inducción de la apoptosis y la inhibición de la proliferación celular dependiente de la dosis. El acoplamiento calculado y el método DFT sugieren una relación estructura-actividad entre las actividades de la dimetoxicurcumina y la estructura de la curcumina y la apoptosis en las células HepG2.

Palabras clave: curcumina, hepatocarcinoma

RESUM:

Es van estudiar les relacions entre les característiques estructurals de la curcumina i la dimetoxicurcumina i la seva activitat antitumoral. El tractament de les cèl·lules HepG2 durant 24 hores amb la curcumina i la dimetoxicurcumina va donar lloc a la inducció de l'apoptosi i la inhibició de la proliferació cel·lular depenent de la dosi. L'acoblament calculat i el mètode DFT suggereixen una relació estructura-activitat entre les activitats de la dimetoxicurcumina i l'estructura de la curcumina i l'apoptosi a les cèl·lules HepG2.

Paraules clau: curcumina, hepatocarcinoma

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INTRODUCTION

The discovery of Curcumin dates around the nineteenth centuries when Vogel reported the isolation of "a yellow colouring-matter" from the rhizomes of *Curcuma longa*. Later, this substance of yellow colour was found to be a mixture of resin (hydrocarbon secretion of many plants) and turmeric oil. However, only until 1910 the group of Milobedzka and Lampe identified the chemical structure of Curcumin as diferuloylmethane or (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (IUPAC name). The same group in 1913 carried out the synthesis of the compound, with very low yield for the reaction. [1–3]

Although turmeric, the major source of Curcumin, has been consumed as a dietary spice with human food for thousands of years around the world, the biological characteristics of Curcumin and some of its derivatives were not identified neither studied until the midd-20th century [3,4]. Despite those findings, few papers were published on the field of Curcumin during the next two decades, due to the very difficult synthesis procedure of the pure compound. The Curcumin and several analogs became the main subject of scientific research and there were discovered diverse biological properties of Curcumin, including anticancer activity of Curcumin using *in vitro* and *in vivo* models. [5–8]

As it was said, this kind of compounds have a huge amount of pharmacological activities and extremely superior safety profile, but the administration of Curcumin to a human patient has a very important and unsolved practical problem. Measurements of blood plasma levels and biliary excretion showed that Curcumin was poorly absorbed from the intestine and the quantity of Curcumin that reached tissues outside the intestine was pharmacologically insignificant, and therefore there was not important benefits of Curcumin to induced diseases in rats. The studies indicated that the main failure of Curcumin was its poor solubility in water at physiological pH, limited absorption through the intestine walls, poor bioavailability, fast metabolism in the liver and fast excretion through the feces or urine. Therefore, to see the therapeutic effects of Curcumin in the human body, making the parallel from the rats, an average person should be required to swallow between 12 and 20 g of Curcumin per day; otherwise, it is very unlikely that a measurable concentration of Curcumin occurs in the body after ingestion. This amount of Curcumin would be very expensive to synthesize, and therefore alternative ways to get the Curcumin or related compounds into the human body are required. Another way to get the Curcumin into the blood plasma is to administrate it with other substances that enhance the solubility of Curcumin, but this decreases its biological activity . [5,9–11]

Furthermore, there are several preclinical and clinical trials in humans which showed the same issues, such as poor bio-availability, fast metabolism and requirement of reiterative and very high oral dosages. However, Curcumin is today an excellent starting compound for drug design and development of new drugs, on the basis of explicit bio-activities, non-toxicity and accessible synthesis, which is an area not deeply explored nowadays. [7,8,12–15]

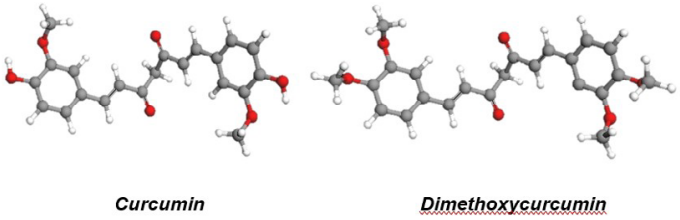


Figure 1: *Molecular structures of the curcumin and dimethoxycurcumin.*

Recent works indicate that Curcumin and Dimethoxy curcumin induced a cytotoxic stimulus in the 786-O renal tumor cell line, and the structural modifications in each molecule influenced their mechanism of action.[16]

In this paper we propose to determine of biological activity of a Curcumin and Dimethoxy Curcumin. To get a complete study of the compounds, we also will use computational chemistry to carry out the theoretical characterization. Furthermore, it is well known that Curcumin and Dimethoxy Curcumin [Figure 1] have interesting biological activities; therefore, we want to pursue initial biological activity determinations of these compounds, antiproliferative activity determination in HepG2 cells.

EXPERIMENTAL SECTION

Cell culture

Human hepatocellular carcinoma HepG2 cells (American Type Culture Collection HB-8065) [17] were grown in monolayer culture in DMEM-high glucose with 10% FBS and antibiotic-antimycotic (GIBCO). All cell cultures were grown at 37 °C in a 5%: 95% CO_2: air atmosphere controlled.

Cell viability assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Invitrogen, Eugene, Oregon, USA). Cell viability was quantified by the amount of MTT reduction [18] HepG2 were exposed to different concentrations of curcumins for 48 hours. After treatment cells were co-incubated with MTT (0.5 mg mL) for 4 hours, and then solubilized with an acidified (0.04 N HCl) isopropanol/dimethyl sulfoxide (DMSO) solution. Curcumin, and MethoxyCurcumin were purchased from Sigma–Aldrich. Optical density was measured at 540 nm. All experiments were performed as triplicates. Data were expressed as percentage of survival cell. Cell survival (%) data were plotted and adjusted to a sigmoidal best-fit curve, where: IC_{50} is the curcumins concentration to reach the half-maximal cell survival.

Internucleosomal DNA fragmentation assay

DNA was extracted from cells as described previously [19]. Fragmented DNA samples were separated by electrophoresis on 1.5 agarose gel and visualized with ethidium bromide.

Western blot

HepG2 cells were lysed in cold lysis buffer, and then proteins were extracted. Supernatants were collected and stored in the same lysis buffer. Protein extract and supernatant were subjected to SDS-PAGE and resolved proteins were transferred to a nitrocellulose or poly vinylidene fluoride (PVDF) membrane. The blocked membrane was incubated with the appropriate primary antibody two hours or overnight (ON), washed twice, and incubated with a secondary antibody two hours or ON. Primary antibodies HSP70 (1:5000, mouse, Origene, MD, USA) was used as a loading control and Caspase-3 (1:1000, rabbit, Cell Signaling, MA, USA). Secondary antibodies mouse and rabbit, 1:5000 Cell Signaling, MA, USA and bands were revealed using a peroxidase-conjugated IgG antibody.[20]

Determination of Intracellular ROS

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable probe for intracellular ROS, is converted into DCF-DA by intracellular esterase's and rapidly oxidized to the highly fluorescent DCF in the presence of intracellular hydrogen peroxide and peroxidases. The treated cells were incubated for 30 min with 10 μM DCFH-DA and washed with PBS. DCF fluorescence was detected by a fluorescence plate reader (Tecan infinite® m200pro). ROS levels were expressed as the normalized fluorescence unity's (RFU) of DCF.[21]

Data analysis

These data represent the average ± SEM of at least three independent experiments performed in triplicate. Statistical differences were assessed by a one-way ANOVA (Kruskal-Wallis) followed by Dunn`s *post hoc* test

Molecular modeling

The DFT theoretical calculations were carried out using Amsterdam Density Functional (ADF) code [22]. All the real cluster structures were fully optimized via analytical energy gradient method implemented by Verluis and Ziegler employing the local density approximation (LDA) within the Vosko-Wilk-Nusair parametrization for local exchange correlations. We also used the GGA (Generalized Gradient Approximation) BLYP functional [23].

Solvation effects were modeled by a conductor-like screening model for real solvents (COSMO) [19] using dichloromethane as solvent. The cluster geometry optimization and the excitations energies were calculated using a standard Slater-type-orbital (STO) basis sets with triple-zeta quality double plus polarization functions (TZ2P) for the all the atoms [16]. We carried out harmonic frequency calculations on each cluster to confirm that the converged geometries were equilibrium structures.

Configurational optimization and prediction of the free energy of the interactions involved in DNA/Drugs that have multiple degrees of freedom can be approximated satisfactorily in the complex systems. The simplified global systems were calculated computationally by means of an empirical function of points (Score) and a coupling vector (shape of a spring) in the ArgusLab program [24] with a Dock coupling protocol (score/shape), so that all the energy minima of the simulated systems are explored formally [25].

The AScore is based on the deconvolution of the total DNA-ligand binding free energy into different components:

$$\Delta G_{binding} = \Delta G_{vdW} + \Delta G_{hydrophobic} + \Delta GH\text{-bond} + \Delta GH\text{-bond}_{(chg)} + \Delta G_{deformation} + \Delta G^{\circ}$$

The dissected terms account for the van der Waals interaction between the ligand and the DNA (ΔG_{vdW}), the hydrophobic effect ($\Delta G_{hydrophobic}$), the hydrogen bonding between the ligand and the protein ($\Delta G_{hydrophobic}$), the hydrogen bonding involving charged donor and/or acceptor groups ($\Delta G_{H\text{-}bond(chg)}$), the deformation effect ($\Delta G_{deformation}$), and the effects of the translational and rotational entropy loss in the bidding process (ΔG°).

RESULTS AND DISCUSSIONS

Dimethoxycurcumin being a derivative of curcumin was checked for its ability to induce toxicity in one tumor cell line, HepG2 at 24 h after treatment. For dimethoxycurcumin comparison, the cytotoxicity studies of curcumin were also performed under similar experimental conditions. In HepG2, both Dimethoxycurcumin and curcumin induced cytotoxicity, which increased with increasing treatment concentration from 5 to 100 μM (Fig. 2). This suggested that Dimethoxycurcumin like curcumin could show toxicity to tumor cells., these can be considered as promising candidates for chemotherapy drugs, particularly the molecule with the highest potency, Dimethoxycurcumin.[16]

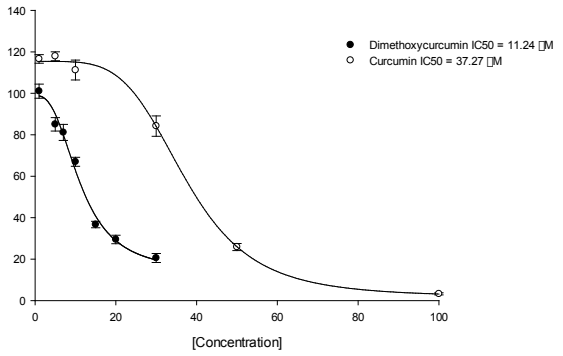


Figure 2: *Effects of curcumin and dimethoxycurcumin on the growth of HepG2 hepatome cells after 24 h Incubation. The results of studies are expressed as mean values ±SEM from three separate experiments (p<0.05).*

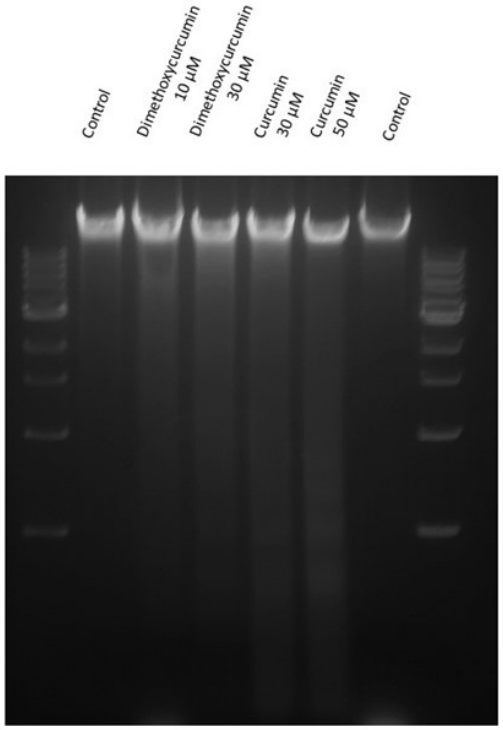


Figure 3: *Effects of curcumin and dimethoxycurcumin on the DNA fragmentation pattern analysis by agarose gel electrophoresis of HepG2 cells after 24 h incubation.*

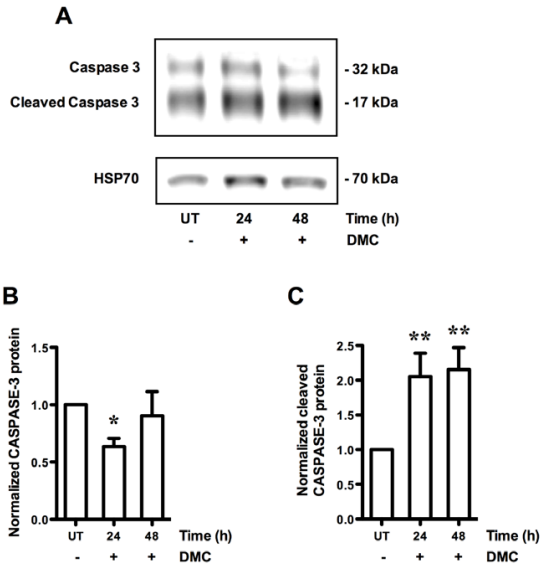


Figure 4. Dimethoxycurcumin-induced caspase pathway in HepG2 cells. Cells were treated with DMC (10 µM) for 24 and 48 hours, and protein expression was analysed. (**A**) representative images from western blot experiments performed for the detection of caspase-3 and cleaved caspase-3. (**B** and **C**) Densitometric analyses of the experiments shown in **(A)**. Protein levels were normalyzed againts HSP70 and data are expressed relative to the UT (untreated) condition. Statistical differences were assessed by a one-way ANOVA (Kruskal-Wallis) followed by Dunn`s *post hoc* test. **P*<0.05; ***P*<0.001 vs. UT group.

Since DNA fragmentation is a classical sign of apoptosis, [20] internucleosomal DNA fragmentation was evaluated in HepG2 cells previously exposed to the curcumin and dimethoxycurcumin using DNA laddering assay. As shown in Fig. 3, typical apoptosis ladder DNA patterns were observed in HepG2 cells exposed to 30 µM and 50 µM of curcumin and dimethoxycurcumin respectively, a dose close to IC_{max}, suggesting that exposure to the curcumin and dimethoxycurcumin may induce cell apoptosis. As expected, exposure to a dose lower than IC_{50} decreased apoptotic DNA patterns to levels barely perceptible.

Caspase proteins play a major role in the activation of apoptosis. For this reason, activation by caspases-3 was analyzed in HepG2 cells treated with DMC.

The total amount of the Caspase-3 protein decreased significantly (P<0.05) at 24 h (Figure 4A and B), whereas the total cleaved caspase-3 protein level significantly increased (P<0.01) at 24 and 48 h in HepG2 cells treated with DMC (Figure 4A and C). Increased active caspase-3 (cleaved caspase-3) suggests activation of apoptotic endonuclease, is a key enzyme that mediates regulated DNA fragmentation and chromatin condensation in response to apoptotic signals[26]

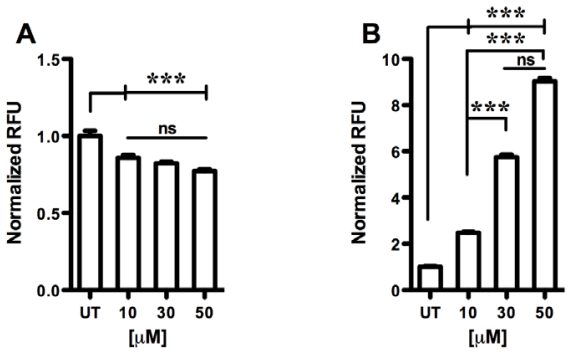


Figure 5. **Effects of Curcumin and Dimethoxycurcumin on HepG2 cells intercelular reactive oxygen species generation**. Cells were exposed to CUR (**A**) and DMC (**B**) at different concentrations for 4 h. Cells were stained with DCFDA and analysed by fluorescence plate reader (Tecan infinite® m200pro). Data are expressed as the mean ± SEM from three independent experiments, each performed in triplicate. Statistical differences were assessed by a one-way ANOVA (Kruskal-Wallis) followed by Dunn`s *post hoc* test. ****P*<0.0001. UT: Untreated group; ns: non significant.

To analyze the levels of ROS in HepG2 cells treated with CUR and DCM, we used a fluorescent probe DCFDA. ROS levels were observed in HepG2 cells treated with both compounds, for 4 hours. As Fig. 5A shows, a decrease of RFU (Relative Fluorescence Unit) cells treated with CUR was observed; RFU decreased significantly (P<0.0001) at different concentration 10, 30 and 50 µ M compared to untreated (UT) condition. Statistical analysis reveals no significant differences between at diffrent concentration: While cells treated with DMC significantly (P<0.0001) increases the RFU at different concentration 10, 30 and

50 µM compared to UT condition. Moreover, comparing 10 and 30 µ M and 10 and 50 µ M show significant mean difference (P<0.0001) and 30 and 50 µ M were not significant mean difference.

Previous results are indicated that the differential apoptotic activities of dimethoxycurcumin and curcumin are positively associated with the extent of stability of these two compounds. However, it is also possible that curcumin is a chemopreventive rather than hemotherapeutic agent for cancer, whereas, reversibly, dimethoxycurcumin is primarily a tumor chemotherapeutic rather than chemoprotective agent. [27].

The simulation, conceived as a complementary and synthetic model, optimizes structurally in a representative manner the experimental systems. The fact of inhibiting the replication process of DNA by action of drugs is the motivation of designing new and more efficient antitumor drugs. Moreover, their effectiveness depends on the mode and affinity of their binding ability to the DNA strands.[28].

According to this docking experiment, the complexes reasonably bind with DNA. The minimum energy obtained for a docked structure (Figure 6) suggest that the best possible conformation of the ligand interaction, is mainly through the aromatic ring being inside the DNA strand. It has been observed that the complex is stabilized by electrostatic hydrogen bonding with DNA bases, in addition to van der Waal's and stacking–bond interactions between electron deficient chalcone ring and purine–pyrimidine bases. The binding energy values are -4,03523 kcal/mol and 3,94258 kcal/mol of curcumin and dimethoxycurcumin respectively. The HOMO and LUMO orbitals are commonly known as Frontier Orbitals and were found to be extremely useful in explaining chemical reactivity. Electrophilic attacks were shown to correlate very well with atomic sites having high density of the HOMO orbital, whereas nucleophilic attacks correlated very well with atomic sites having high density of the LUMO orbital. The molecular frontier orbitals show differences between the dimethoxycurcumin and curcumin, [Figure 7] where in the case of the dimethoxycurcumin are centered in the methoxy group and change in Curcumin are localized on the diketone region; This would agree with the described by Kunwar (Kunwar et al., 2011) and colleagues were indicated that dimethoxy curcumin is more stable towards hydrolysis and chemical oxidation than curcumin.

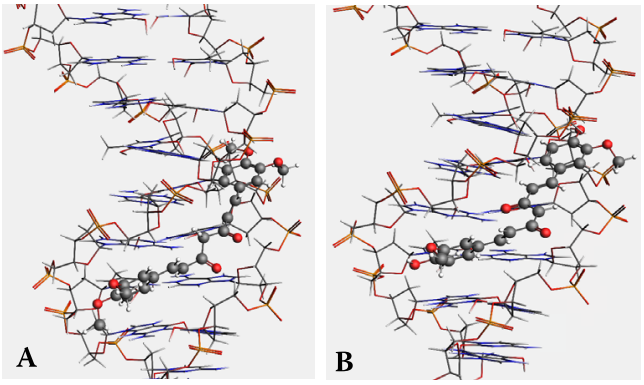
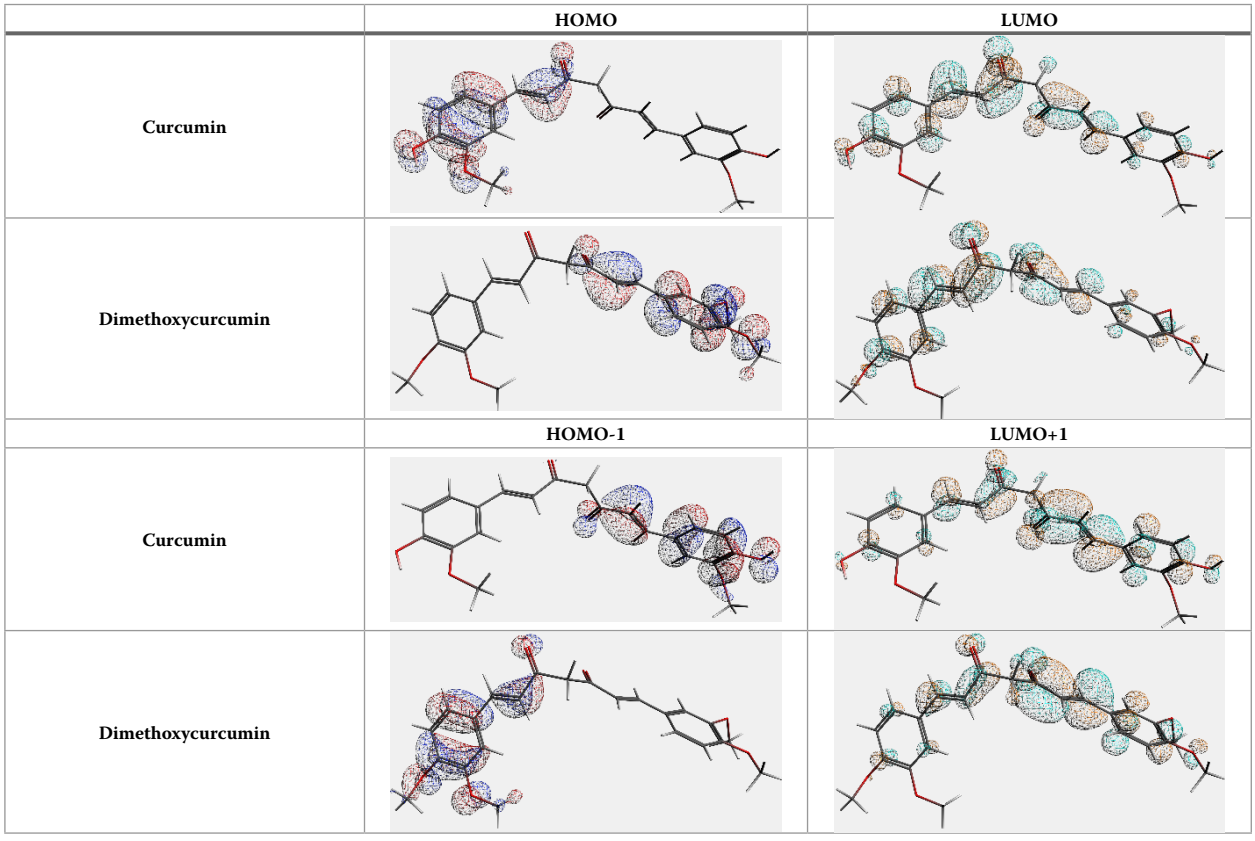


Figure 6: *Molecular simulations of the curcumin(A) and dimethoxycurcumin(B) with DNA*

Figure 7: *Molecular orbital of the curcumin and dimethoxycurcumin.*



CONCLUSIONS

Treatment of HepG2 cells for 24 h with curcumin and dimethoxycurcumin resulted in a dose dependent inhibition of cell proliferation and apoptosis induction. The calculated molecular orbitals using the DFT method and docking calculations indicated the presence of interactions between the curcumin and Dimethoxycurcumin with DNA, which could explain the differences in cytotoxicity obtained suggest a structure-activity relationship between the activities of dimethoxycurcumin and curcumin structure and the apoptosis in HepG2 cells.

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